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(54) Title: A METHOD FOR THE PREPARATION OF A PHOSPHOLIPID WITH A CARBOXYLIC ACID RESIDUE IN THE 2-POSITION AND A PHOSPHOLIPID WITH AN ω-3-FATTY ACID RESIDUE IN THE 2-POSITION

(57) Abstract

The present invention relates to phospholipids with a desired carboxylic acid residue, such as an ω-3-fatty acid residue, in the 2-position. These compounds are produced by esterifying a conventional lysophospholipid with the corresponding carboxylic acid in the presence of the catalyst phospholipase A2, the esterification taking place in a microemulsion with a water content not exceeding 0.1-2 % by weight.

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would lead to the same type of qualitative changes as for the triglycerides.

Recently, medical interest has focussed on the ω-3-fatty acids which is the generic term for polyunsaturated fatty acids which have 18-22 carbon atoms and whose last double bond, as counted from the carboxyl group, is between the third and the fourth carbon atom as counted from the methyl group end of the fatty acid molecule. A connection has been shown between a high intake of ω-3-fatty acids and a reduced frequency of heart and vascular diseases. An augmented intake of ω-3-fatty acids reduces the cholesterol content of the blood, and ω-3-fatty acids are therefore often prescribed for people with blood counts indicating an increased risk of thrombosis and infarct of the heart. The ω-3-fatty acids are normally available not only in the form of triglycerides from e.g. cod-liver oil, but also in the form of free fatty acids usually extracted from fish oils. In the human body, the triglycerides are metabolised, and part of the fatty acids are incorporated in the cell membranes of the body, a main component of these membranes being phospholipids. However, this incorporation is a slow process and only a minor amount of the added ω-3-fatty acids is incorporated in the membranes, regardless of whether they originally had the form of triglycerides or free fatty acids. Therefore, there is a great need for products which contain ω-3-fatty acids and can be taken up by the body in a more efficient manner.

With the aid of a specific enzyme, phospholipase A2, it has now proved to be feasible to esterify the 2-position of a lysophospholipid by adding a carboxylic acid. Normally, phospholipase A2 hydrolyses the ester bond of the phospholipid in the 2-position, but under the conditions prevalent during the inventive esterfication, the enzyme esterifies a lysophospholipid in the 2-position, surprisingly enough. This reaction takes place in a micro-emulsion. Since the phospholipid is surface-active in

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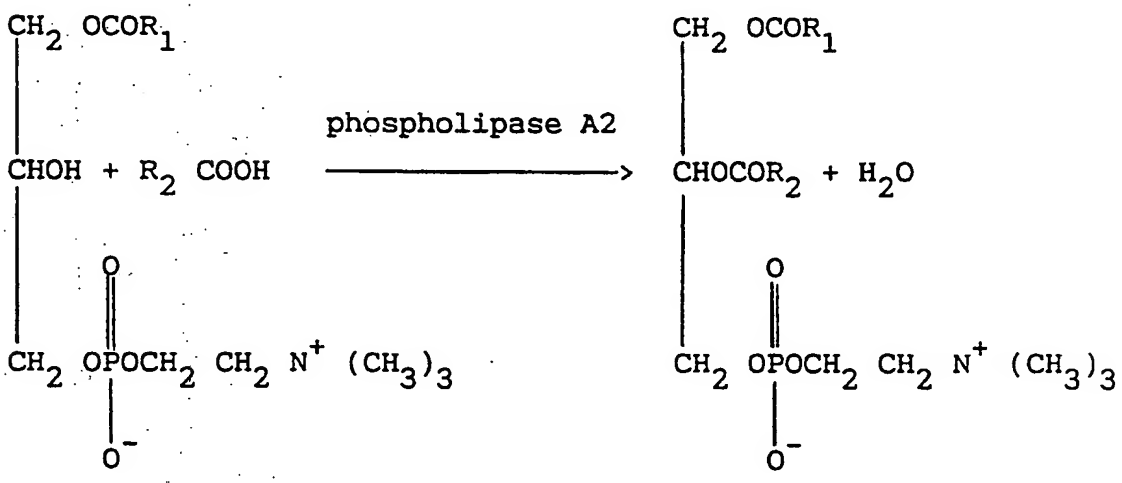
group derived from phosphoric acid and a nitrogen base. Phosphatidyl choline is usually a main component. Furthermore, there are varying amounts of several closely-related substances, such as lysophosphatidyl ethanolamine, lysophosphatidyl serine, and lysophosphatidyl inositol. Generally, the added ω-3-fatty acid is not pure, but consists of a mixture of different fatty acids, such as EPA and DHA, and further contains a fairly significant amount of fatty acids other than the ω-3-type. Even if pure ω-3-fatty acid were to be used in the inventive reaction, the incorporation in the phospholipid would not be complete, since the esterification is an equilibrium reaction. All in all, the ω-3-fatty acid-containing phospholipid referred to in this context may consist of a large number of different substances. However, a distinctive feature is that a fairly significant proportion of the phospholipid, i.e. at least 10% and usually 15% or more, contains ω-3-fatty acid in the 2-position.

If desired, the inventive reaction may be illustrated by the reaction formula of lysophosphatidyl choline.



wherein R_1 is an acyclic hydrocarbon residue which contains 11-21 carbon atoms and is not being of ω-3-type, and R_2 is a polyunsaturated ω-3-fatty alkyl group with 17-21 carbon atoms.

to form a single process in which phospholipid and carboxylic acid together with an enzyme are added to a microemulsion with the higher water content and in which the water content is gradually reduced by stripping under vacuum, or by adding a hydrophilic substance, e.g. zeolite. Even if the water content is not varied, a certain amount of phospholipid containing the desired carboxylic acid residue in the 2-position can be obtained, but the yield is usually poor and the reaction time long.

Preferably, the reaction is made to take place in a proctective atmosphere and in the presence of an antioxidant in order to avoid autoxidation of the polyunsaturated fatty acids. Suitable antioxidants include tocopherol, butyl hydroxyanisole, butyl hydroxytoluene, and ascorbic acid. Combinations of at least one lipophilic and at least one hydrophilic antioxidant have at times proved advantageous.

The invention will be illustrated in more detail by the following Examples.

Example 1

The following composition was used:

Component	% by weight
Isooctane	87.3
Sodium dioctyl sulphosuccinate	3.4
Lysophosphatidyl choline	4.0
ω-3-fatty acid	4.0
Aqueous buffer, pH 8.2	1.3

To the above composition which, at 30°C, was a limpid isotropic solution, was added phospholipase A2 in an amount of $2.5 \cdot 10^4$ units/g lysophospholipid. The reaction was allowed to continue at 30°C under N_2, the solution being continuously stirred. After 16 h, the reaction was interrupted. The phospholipid was isolated by chromatography on a silica column, and the fatty acids were set free by hydrolysis and methylated, whereupon the esters were analysed by gas chromatography. The 10-metre silica columns used in the gas chromatography had an inner dia-

To this composition, $CaCl_2$ was added to a concentration of 10 mM and phospholipase A2 in an amount of $1.5 \cdot 10^4$ units/g phosphatidyl choline. The reaction was allowed to continue for 16 h at 30°C, whereupon the water content was reduced to 1.5% by weight by an addition of zeolite. ω-3-fatty acid in an amount corresponding to 6% by weight of the composition was added. After a further 16 h at 30°C, the reaction mixture was processed as in Example 1. The amount of phospholipid containing ω-3-fatty acid residues was found to be 58% by weight.

Example 5

The following composition was used:

Component	% by weight
Isooctane	87.3
Sodium dioctyl sulphosuccinate	3.4
Lysophosphatidyl choline	5.0
Dodecanoic acid	3.0
Aqueous buffer, pH 8.2	1.3

To the above composition which, at 30°C, was a limpid isotropic solution, was added phospholipase A2 in an amount of $2.5 \cdot 10^4$ units/g lysophospholipid. The reaction was allowed to continue at 30°C under N_2, the composition being continuously stirred. After 16 h, the reaction was interrupted. The phospholipid was isolated by chromatography on a silica column, and the fatty acids were set free by hydrolysis and methylated, whereupon the esters were analysed by gas chromatography. The 10-metre silica columns used in the gas chromatography had an inner diameter of 0.32 mm, Carbowax 1.2 µm serving as a stationary phase. Nitrogen gas under a pressure of 5 psi and a flow rate of 120 ml/min. was used as carrier gas. The column temperature was 275°C. With the aid of the gas chromatogram, the amount of phospholipid containing a dodecyl group was determined to more than 90% by weight. The reaction of lysophospholipid gave a 12% yield of phospholipid.

CLAIMS

1. A method for the preparation of a phospholipid with a carboxylic acid residue in the 2-position, characterised in that a lysophospholipid is esterified with a corresponding carboxylic acid in the presence of the catalyst phospholipase A2, the esterification taking place in a microemulsion with a water content of 0.1-2% by weight.

2. Method as claimed in claim 1, characterised in that the carboxylic acid is an aliphatic carboxylic acid with 10-22 carbon atoms.

3. Method as claimed in claim 1 or 2, characterised in that the carboxylic acid is an ω-3-fatty acid.

4. Method as claimed in any one of claims 1-3, characterised in that the surface-active component of the microemulsion comprises, apart from the lysophospholipid, at least one nonionic or anionic surface-active compound, or mixtures thereof, in an amount of 0.1-10% by weight of the total composition, and that the hydrophobic component of the microemulsion constitutes 65-98% by weight of the total composition.

5. Method as claimed in claim 3 or 4, characterised in that the lysophospholipid and the ω-3-fatty acid are added in an amount of 1-20% by weight of the total composition.

6. Method as claimed in any one of claims 3-5, characterised in that the ω-3-fatty acid contains 18-22 carbon atoms.

7. Method as claimed in any one of claims 1-6, characterised in that the lysophospholipid is largely made up of lysophosphatidyl choline, lysophosphatidyl ethanolamine, lysophosphatidyl serine and lysophosphatidyl inositol, or mixtures thereof.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 90/00481

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 7/64, C 11 C 3/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 11 C; C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Chemical Abstracts, Vol. 90 (1979), abstract No 50071c, R. W. Evans et al. Chem. Phys. Lipids 1978, 22(3), 207-20 (Eng.) --	9
Y	Chemical Abstracts, Vol. 73 (1970), abstract No 84125n, H. P. Franck et al., Z. Naturforsch B 1970, 25(6), 581-6 (Ger.) --	1-8
Y	Chemical Abstracts, Vol 69 (1968), abstract No 168k, H. P. Franck et al. Z. Naturforsch. B 1968, 23(4). 43948 (Ger.) --	1-8
Y	Chemical Abstracts, Vol. 82 (1975), abstract No 53462b, Ronald L. Misiorowski et al., Biochemistry 1974, 13(24), 4921-7 (Eng.) --	1-8

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th October 1990	1990 -10- 15
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Yvonne Siösteen Yvonne Siösteen

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 90/00481

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 90-08-28
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0265699	88-05-04	JP-A- 63087988	88-04-19
SE-B- 452166	87-11-16	EP-A-B- 0237092	87-09-16
		SE-A- 8601222	87-09-11
		US-A- 4839287	89-06-13
FR-A1- 2599382	87-12-04	NONE	